Human Superoxide Dismutase 3 ELISA

Cat. No.: RLF-EK0107R

1. Introduction

Superoxide dismutase (SOD) is an antioxidative enzyme involved in the defense system against reactive oxygen species (ROS). SOD catalyzes the dismutation reaction of superoxide radical anion (O_2^-) to hydrogen peroxide, which is then catalyzed to innocuous O_2 and H_2O by glutathione peroxidase and catalase. Three unique and highly compartmentalized mammalian superoxide dismutases have been biochemically and molecularly characterized to date. SOD1, or CuZn-SOD (EC 1.15.1.1), was the first enzyme to be characterized and is a copper and zinc-containing homodimer that is found almost exclusively in intracellular cytoplasmic spaces. SOD2, or Mn-SOD (EC 1.15.1.1), exists as a tetramer and is initially synthesized containing a leader peptide, which targets this manganese-containing enzyme exclusively to the mitochondrial spaces. SOD3, or EC-SOD (EC 1.15.1.1), is the most recently characterized SOD, exists as a copper and zinc-containing tetramer.

SOD3, which is highly expressed in selected tissues including blood vessels, heart, lungs, kidney and placenta, is found in the extracellular matrix of tissues and is ideally situated to prevent cell and tissue damage initiated by extracellularly produced ROS. SOD3 contains a unique heparin-binding domain at its carboxy-terminus that establishes localization to the extracellular matrix where the enzyme scavenges superoxide anion. SOD3 plays an important role in maintaining vascular tone, attenuating age-related cognitive decline, lung function, and the metabolism of NO, and in the pathology of such diseases as atherosclerosis, diabetes, and arthritis.

2. Principles of Method

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human SOD3. Samples are pippetted into these wells. Unbound SOD3 and other components of the sample is removed by washing, then biotin-conjugated monoclonal antibody specific to SOD3 is added. In order to quantitatively determine the amount of SOD3 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well. Next, a TMB-substrate solution is added to each well. Finally, a sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. The absorbance (O.D. value) is directly proportional to the amount of captured SOD3.

3. Intended Use

The AbFrontier human Superoxide Dismutase-3 (human SOD3) ELISA kit is to be used for the in vitro quantitative determination of human SOD3 in cell lysate and buffered solution. The assay will recognize both native and recombinant human SOD3.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. Storage and Stability

All kit components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. Chemical Hazard

- Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- All reagents containing Sodium Azide also contain Thimerosal as a preservative. Thimerosal contains Hg and thus should be handled with great care.

6. Kit Contents

Contents	Number	Volume
96 Well Plate	1 (in aluminum foil bag with desiccant)	
Incubation Buffer	1	30 ml
Washing Buffer	1	(10X) 100 ml
Standard Protein	1 Glass vial (lyophilized)	
Standard/Sample Dilution Buffer	1	25 ml
Secondary Antibody	1 Glass vial (lyophilized)	
AV-HRP	1	(100X) 150 µl
Secondary Antibody/AV-HRP Dilution Buffer	1	25 ml
Substrate (TMB)	1	20 ml
Stop Solution	1	20 ml
Protocol booklet	1	
Plate sealers	3	

① 96 Well Plate

: Human SOD3 microtiter plate, one plate of 96 wells (16well strip x 6).

A plate using break-apart strips coated with a mouse monoclonal antibody specific to human SOD3.

② Standard Protein

: Recombinant human SOD3.

③ Secondary Antibody

: Biotin labeled mouse anti human SOD3 antibody.

④ AV-HRP

: Avidin linked Horseradish Peroxidase (HRP, enzyme)

⑤ Substrate (Stabilized chromogen)

: Tetramethylbenzidine(TMB) solution

⑥ Stop Solution

: 1N solution of sulfuric acid (H_2SO_4).

⑦ Plate sealer

: Adhesive sheet.

- Do not mix or interchange reagents from different lots.

7. Materials Required But Not Provided

① Microtiter plate reader capable of measurement at or near 450nm.

② Calibrated, adjustable precision pipettes, preferably with disposable plastic tips (A manifold multi-chan nel pipette is desirable for large assays.)

③ Distilled or deionized water

④ Data analysis and graphing software

⑤ Vortex mixer

⑥ Polypropylene tubes for diluting and aliquoting standard

⑦ Absorbent paper towels

⑧ Calibrated beakers and graduated cylinders of various sizes

8. Reagent Preparation

1) Human SOD3 standard

First, make the working standard protein solution by mixing S*tandard/Sample Dilution Buffer* 1ml with *Standard Protein Stock Solution* 1µl, which will be 1µg/ml.

Standard	Add	Into
128 ng/ml	128 µl of the 1 µg/ml working std. solution	872 µl of the *Standard/Sample Dilution Buffer*
64 ng/ml	64 µl of the 1 µg/ml working std. solution	936 µl of the *Standard/Sample Dilution Buffer*
32 ng/ml	32 µl of the 1 µg/ml working std. solution	968 µl of the *Standard/Sample Dilution Buffer*
16 ng/ml	16 µl of the 1 µg/ml working std. solution	984 µl of the *Standard/Sample Dilution Buffer*
8 ng/ml	8 µl of the 1 µg/ml working std. solution	992 µl of the *Standard/Sample Dilution Buffer*
4 ng/ml	4 µl of the 1 µg/ml working std. solution	996 µl of the *Standard/Sample Dilution Buffer*
2 ng/ml	2 µl of the 1 µg/ml working std. solution	998 µl of the *Standard/Sample Dilution Buffer*
0 ng/ml	1.0ml of the *Standard/Sample Dilution Buffer*	

2) Secondary Antibody

100X secondary antibody solution can be made by adding 150 µl secondary antibody/AV-HRP dilution buffer in the vial.

1. Equilibrate to room temperature, mix gently.

2. Mix 20µl *Secondary Antibody concentrated solution* (100X) + 2ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more if needed) Label as "Working Secondary antibody Solution".

3. Return the unused *Secondary Antibody concentrated solution* to the refrigerator.

3) AV-HRP

1. Equilibrate to room temperature, mix gently.

2. Mix 20 µl *AV-HRP concentrated solution* (100X) + 2 ml *Secondary Antibody/AV-HRP dilution buffer*. (Sufficient for one 16-well strip, prepare more as needed). Label as "Working AV-HRP Solution".

3. Return the unused *AV-HRP concentrated solution* to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.

2. Mix 1 volume *Wash buffer concentrate solution* (10X) + 9 volumes of deionized water. Label as "Working Washing Solution".

3. Store both the concentrated and the Working Washing Solution in the refrigerator.

*** Directions for washing**

1. Fill the wells with 300 µl of "Working Washing Buffer".

Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.

If using an automated washer, the operating instructions for washing equipment should be carefully followed.

2. Incomplete washing or residual wash buffer in wells will adversely affect the assay and render false results.

3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

9. Assay Procedure

- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
- All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
- A standard curve must be run with each assay.
- If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
- Maintain a consistent order of sample and reagent additions from well to well. This ensures equal incubation times for all wells.

1) Determine the number of 16-well strips needed for assay. Insert these in the flame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).

2) Add 300 µl of *Incubation buffer* to all wells and incubate the plate for 5 minutes at room temperature.

3) Thoroughly aspirate or decant the solution from the wells. Wash wells 2 times (See "Directions for washing").

4) For the standard curve, add 100 µl of the standard to the appropriate microtiter wells. Add 100 µl of the *Standard/Sample Dilution Buffer* to zero wells.

5) Add 100 µl of diluted samples to each wells.

6) Cover the plate with the plate cover and incubate for 2 hours at room temperature.

7) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").

8) Pipette 100µl of "Working Secondary Antibody Solution" into each well.

9) Cover the plate with the plate cover and incubate for 1 hour at room temperature.

10) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").

11) Add 100 µl "Working AV-HRP Solution" to each well.

12) Cover the plate with the plate cover and incubate for 30 minutes at room temperature.

13) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See "Directions for washing").

14) Pour enough Substrate you need into a tube or reagent boat. Add 100 µl of *Substrate* to each well. The liquid in the wells should begin to turn blue.

15) Incubate the plate at room temperature. Avoid exposing the microtiterplate to direct sunlight.

- Do not cover the plate with aluminum foil (or other metal), or color may develop.

The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450nm can only be read after the Stop Solution has been added to each well.

- Keep the plate away from direct sunlight because the *Substrate* is light sensitive.
- Typically, reaction is stopped 5~10 minutes after treatment of Substrate, but this time can be adjusted as the user desires.

16) Add 100 µl of *Stop Solution* to each well. The solution in the wells should change from blue to yellow.

17) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the *Sto Solution*.

18) Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and

controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve. 19) Read the human SOD3 concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the S*tandard/Sample Dilution Buffer*).

10. Characteristics

1) Typical result

The standard curve below is for illustration only and **should not be used** to calculate results in your assay.

A standard curve must be run with each assay.

Standard Human SOD3 (ng/ml)	Optical Density (at 450nm)
0	0.094
2.00	0.116
4.00	0.149
8.00	0.210
16.00	0.312
32.00	0.539
64.00	0.951
128.00	1.815

Limitations

- Do not extrapolate the standard curve beyond the 128 ng/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native human SOD3 in various matrices has not been investigated.

2) Sensitivity

The minimal detectable dose of human SOD3 was calculated to be 2 ng/ml, by subtracting two standard deviations from the mean of 10 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

3) Specificity

The following substances were tested and found to have no cross-reactivity: human SOD1, SOD2, SOD4.

4) Precision

① Within-Run (Intra-Assay)

(n=6)

Mean (ng/ml)	SD (ng/ml)	CV (%)
16.81	0.12	0.7
31.83	0.81	2.6
62.40	1.43	2.3
128.64	0.25	0.2

② Between-Run (Inter-Assay)

(n=7)

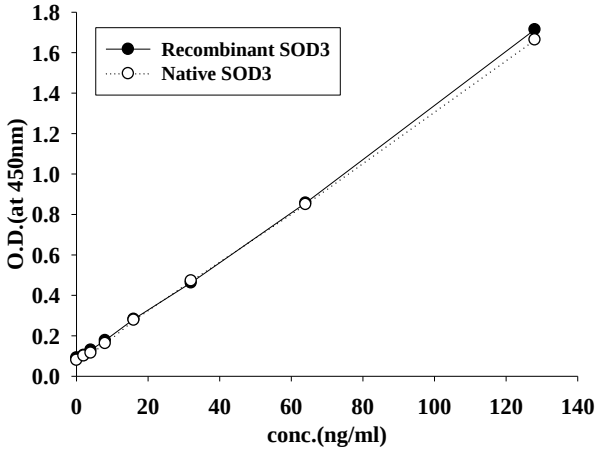
Mean (ng/ml)	SD (ng/ml)	CV (%)
17.44	0.17	1.0
33.62	0.91	2.7
62.93	0.70	1.1
127.89	6.72	5.3

5) Recovery

Recovery upon addition is 95.0~101.9% (mean 98.4%)

Recovery upon dilution is 97.0~105.3% (mean 100.9%)

6) Parallelism



Native human SOD3 from SOD3 transfected cell lysate was serially diluted in sample dilution buffer. The optical density of each dilution was plotted against the recombinant human SOD3 standard curve.

11. Troubleshooting

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash.
	• Too much AV-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30°C before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing – unbound AV-HRP remaining	• Increase number of washes carefully
	• Too much AV-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual AV-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

12 References

1) Steen V. Petersen et al. The dual nature of human extracellular superoxide dismutase: One sequence and two structures (2003) *PNAS* **100**(24):13875-13880

2) Tim D. Oury et al. Human extracellular superoxide dismutase is a tetramer composed of two disulphide-linked dimmers: a simplified, high-yield purification of extracellular superoxide dismutase (1996) *Biochem .J* .**317**:51-57

3) Tohru Fukai et al. Extracellular superoxide dismutase and cardiovascular disease (2002) *Cardiovascular Research* **55**:239-249

4) Yoshiaki Furukawa et al. Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS (2004) *The EMBO Journal* **23**:2872-2881

5) Richard W. Strange et al. The Structure of Holo and Metal-dificient Wild-type Human Cu, Zn Superoxide Dismutase and its Relevance to Familial Amyotrophic Lateral Sclerosis (2003) *J. Mol. Biol*. **328**:877-891

6) Stefan I. Liochev et al. Cross-compartment protection by SOD1 (2005) *Free Radical Biology & Medicine* **38**:146-147

7) Richard A. Weisiger et al. Superoxide Dismutase (1973) *The Journal of Biol. Chem*. **248**(10):3582-3592

8) Igor N. Zelko et al. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD(SOD2), and EC-SOD(SOD3) gene structures, evolution, and expression (2002*) Free Radical Biology & Medicine* **33**(3):337-349

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